

# Formation of Stable Covalent Dimer Explains the High Solubility at pH 4.6 of Lactose- $\beta$ -Lactoglobulin Conjugates Heated near Neutral pH

Saïd Bouhallab,\* François Morgan, Gwénaële Henry, Daniel Mollé, and Joëlle Léonil

Laboratoire de Recherches de Technologie Laitière, INRA,  
65 rue de Saint Brieuc, 35042 Rennes Cedex, France

The solubility of lactose- $\beta$ -lactoglobulin conjugates at pH 4.6, after heating near neutral pH in phosphate buffer/0.116 M NaCl, was investigated by size exclusion chromatography and compared with unmodified protein. Heated conjugates in the temperature range 65–90 °C showed greater solubility at pH 4.6. The proportion of soluble protein increased with the number of bound lactose molecules. Total solubility was obtained for conjugates with nine lactose residues attached per monomer of  $\beta$ -lactoglobulin. The protective effect of bound sugar toward precipitation was associated with the formation of soluble disulfide cross-linked dimers, highly accessible to trypsin digestion. These results suggested that bound lactose, through steric hindrance and high surface hydrophilicity, prevents the thiol-disulfide exchange reactions of the polymerization-aggregation process of lactose- $\beta$ -lactoglobulin conjugates.

**Keywords:** Lactose- $\beta$ -LG conjugates; solubility; heat stability; polymerization

## INTRODUCTION

Whey constitutes a valuable source of proteins with important functional, nutritional, and biological properties. The expression of these properties is, however, largely linked with the physical and chemical modifications that occur during whey processing and protein preparation procedures. Temperature, pH, and ligand binding (sugar, minerals...), by inducing changes in the structure of proteins, are among the most important parameters involved in the alteration of the proteins functionality (Persson and Gekas, 1994).

Heating, the most important treatment in the industrial processing of milk and whey, results in complex structural modifications of proteins. Modifications, such as denaturation, affect protein solubility and, in turn, its foaming, gelation, and emulsification properties (Damodaran, 1997). In the case of whey proteins, the extent of a such heat-induced denaturation is generally assessed by precipitation of denatured-aggregated proteins at pH 4.6 (Roefs and de Kruif, 1994; de Wit and van Kessel, 1996). Probably because of its abundance in whey (i.e., 50% of the total proteins; Nielsen et al., 1996), several studies have been performed on the denaturation-aggregation of  $\beta$ -lactoglobulin ( $\beta$ -LG). The denaturation of this protein is assumed generally to be a process of at least two steps: a partial unfolding of native protein and a subsequent aggregation of denatured molecules via hydrophobic interactions and sulfhydryl-disulfide interchange reactions (Sawyer, 1968).

Numerous studies have reported the effect of the neoglycoconjugates of  $\beta$ -LG on protein aggregation. Attachment of alginic acid oligosaccharide to  $\beta$ -LG was

reported to depress the aggregation process (Hattori et al., 1997). Kitabatake et al. (1985) reported that covalent binding of gluconic or melibionnic acids to  $\beta$ -LG promotes higher solubility. In contrast, Waniska and Kinsella (1989) showed that the solubility of unheated  $\beta$ -LG near pH 4.6 was strongly decreased by the attachment of maltosyl and  $\beta$ -cyclodextrinyl groups.

The formation of neoglycoconjugates between lactose and free amino groups of milk proteins is known to occur at high temperatures or during storage (Matsuda et al., 1991). Recently, we and other researchers have shown a specific glycation of  $\beta$ -LG under mild heat treatment of sweet and acid whey by using electrospray mass spectrometry (Burr et al., 1996; Léonil et al., 1997). In a subsequent work, we have shown that the  $\beta$ -LG monomer is able to covalently bind several lactose molecules, depending on the heat treatment and glycation conditions, that is, aqueous solution versus dry method (Morgan et al., 1997). It was also shown that glycation in aqueous solution led to important structural changes of the association behavior of  $\beta$ -LG, whereas dry method glycation did not significantly alter the natively like state of the protein (Morgan et al., 1999). In the present study we focus on the solubility at pH 4.6, of dry method glycated  $\beta$ -LG after subsequent heat treatment. The solubility of  $\beta$ -LG with an increasing lactose/protein molar ratio, heated from 65 to 90 °C, was investigated using size exclusion chromatography. Furthermore, the biochemical characterization of the soluble species was also carried out.

## MATERIALS AND METHODS

$\beta$ -Lactoglobulin (mixture of A and B variants) was prepared as described by Léonil et al. (1997). The purity of  $\beta$ -LG, assessed by RP-HPLC analysis, was 98%. Monohydrated lactose was obtained from Merck (Darmstadt, Germany).  $\beta$ -LG concentration was determined at 280 nm, using an  $E_{1\text{cm}}^{1\%}$  of

\* Author to whom correspondence should be addressed (fax +33-2-99-28-53-50; e-mail Said.Bouhallab@labtechno.roazhon.inra.fr).

9.6. Tosylphenylalanine chloromethyl ketone treated trypsin (EC 3.4.21.4) was obtained from Serva (Heidelberg, Germany). Soybean trypsin inhibitor (SBTI) and standard proteins, that is, aldolase, human serum albumin, ovalbumin,  $\beta$ -lactoglobulin,  $\alpha$ -chymotrypsinogen, and ribonuclease A, were obtained from Sigma Chemical Co. (St. Louis, MO). Buffers and mobile phases were prepared with HPLC grade water and filtered prior to use. All other reagents were of analytical grade.

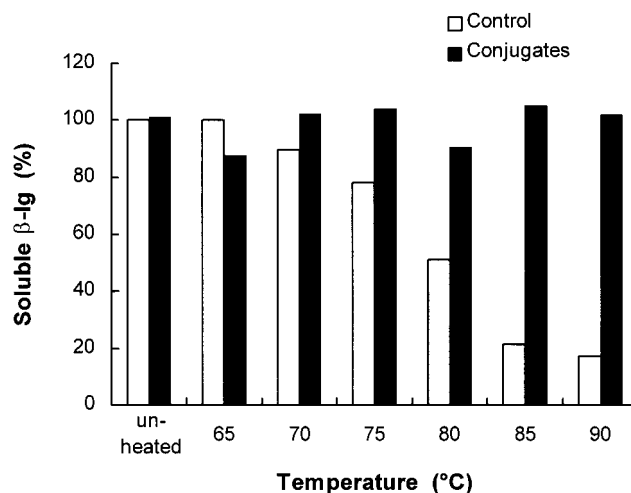
**Glycation Experiments.**  $\beta$ -LG (0.15 mM) was dissolved in a 15 mM lactose solution, and the pH was adjusted to 7.2 with 0.5 M  $\text{NH}_4\text{OH}$ . After freeze-drying, the protein-sugar powders were kept under 65% relative humidity (saturated KI solution) and 50 °C for various periods until 40 h. The temperature was set to 50 °C to promote the Maillard reaction. After such treatment, the powders were dissolved in cooled distilled water to obtain a 0.15 mM  $\beta$ -LG solution, and free lactose was removed by extensive dialysis at 4 °C (50 mL samples in 5 L of distilled water containing 0.02% sodium azide, four water batches in 72 h) in Spectra/Por 1 cellulose membranes (molecular mass cutoff = 6–8 kDa, Spectrum, Laguna Hills, CA). Dialyzed samples were then precipitated at pH 4.6 with 1 M HCl at 23 °C for 1 h and filtered on 0.45  $\mu\text{m}$  filter, and the pH was adjusted to 7.2. All samples were freeze-dried before subsequent use. The average numbers of lactose residues covalently bound per  $\beta$ -LG molecule, as determined according to the method of Morgan et al. (1997), were found to be 1.3, 1.8, 2.5, and 9, respectively, after 5, 6, 10, and 40 h treatment periods. Control samples used in the present study were obtained by incubation of the protein without lactose during the same periods.

**Heat Treatment.**  $\beta$ -LG powders (0.15 mM) with various numbers of bound lactose and corresponding controls were dissolved in 0.1 M phosphate buffer/0.116 M NaCl, pH 7.4. After 1 h of stirring at room temperature, samples were placed in capped flasks and heated in a water bath, at different temperatures, for 10 min. The tubes were then removed and cooled in an ice-water bath.

**Determination of Soluble  $\beta$ -LG at pH 4.6.** Fractions of heated  $\beta$ -LG samples were adjusted to pH  $4.6 \pm 0.1$  with 0.1 M sodium acetate buffer, pH 4. After 1 h at room temperature, the acid-induced precipitation of denatured/aggregated protein was separated by centrifugation for 10 min at 9000g. The residual protein in the supernatant was analyzed by size exclusion chromatography (SEC), with detection at 214 nm. The area of soluble protein is given as a percentage of the total area obtained before heating and acid precipitation. The presented results are the average of two determinations, with a coefficient of variation of <10%. Triplicate determinations were performed on the behavior of the conjugates with nine lactose residues per  $\beta$ -LG molecule, and the corresponding control, after heating at 90 °C, which were selected for further characterization. The determined coefficient of variation was in the same range.

**Tryptic Hydrolysis.** Tryptic hydrolyses were performed on heated samples before and after acid precipitation. For precipitated samples, the supernatants of precipitation at pH 4.6 were first adjusted to pH 8 by dilution in 0.1 M phosphate buffer. Hydrolysis experiments were then performed at 40 °C on  $\beta$ -LG (48  $\mu\text{M}$  in 100 mM phosphate buffer) with trypsin at an enzyme/substrate molar ratio of 1:140. Aliquots were taken at intervals, and the reaction was stopped by adding SBTI to an inhibitor/enzyme ratio of 7 (mole/mole) and by lowering the temperature in an ice-water bath. Hydrolysis was monitored by SEC.

**SEC.** Samples were injected (50  $\mu\text{L}$  for  $\beta$ -LG samples or 100  $\mu\text{L}$  for hydrolysates) onto a Superdex 75 HR column (10 i.d.  $\times$  300 mm, Pharmacia, Uppsala, Sweden) at room temperature. The elution buffer (buffer A) was 0.1 M Tris-HCl, pH 8, containing 0.15 M NaCl. Elution was achieved in isocratic mode at 0.5 mL/min, for 50 min, in an HPLC system fitted with 2600 Chromatography Nelson Analytical software by means of a 900 Series Intelligent Interface (Nelson, Cupertino, CA). The absorbance was monitored at 214 nm. The standard proteins used for calibration were aldolase (158 kDa), human serum albumin (66 kDa), ovalbumin (43 kDa),  $\beta$ -LG (36.6 kDa),



**Figure 1.** Solubility at pH 4.6 of control and lactose- $\beta$ -LG conjugates (nine lactose residues per molecule of  $\beta$ -LG) as a function of heating temperature. Samples (3 mg/mL) were heated for 10 min in 0.1 M phosphate buffer/0.116 M NaCl, pH 7.4.

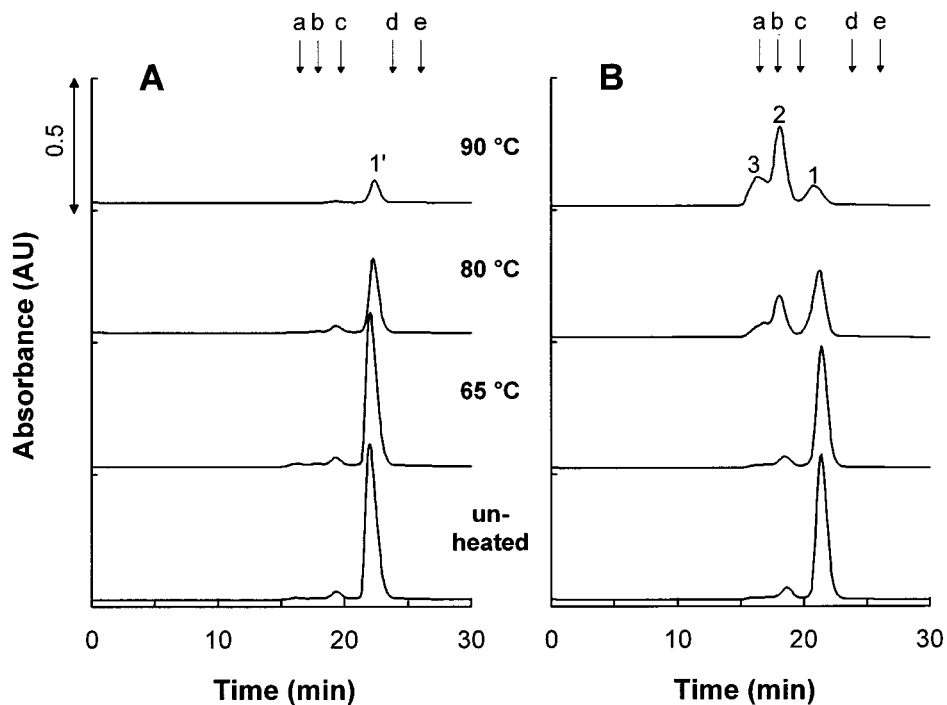
$\alpha$ -chymotrypsinogen (25 kDa), and ribonuclease A (13.6 kDa). The void volume was determined with Blue Dextran 2000.

To determine the nature of interactions involved in the new formed species, heated samples were analyzed in the presence of urea  $\pm$  dithiothreitol (DTT), as reported by Hoffmann et al. (1997). Samples were incubated with buffer B (buffer A, containing 6 M urea) or with buffer C (buffer B containing 10 mM DTT). Elution was performed in buffer B.

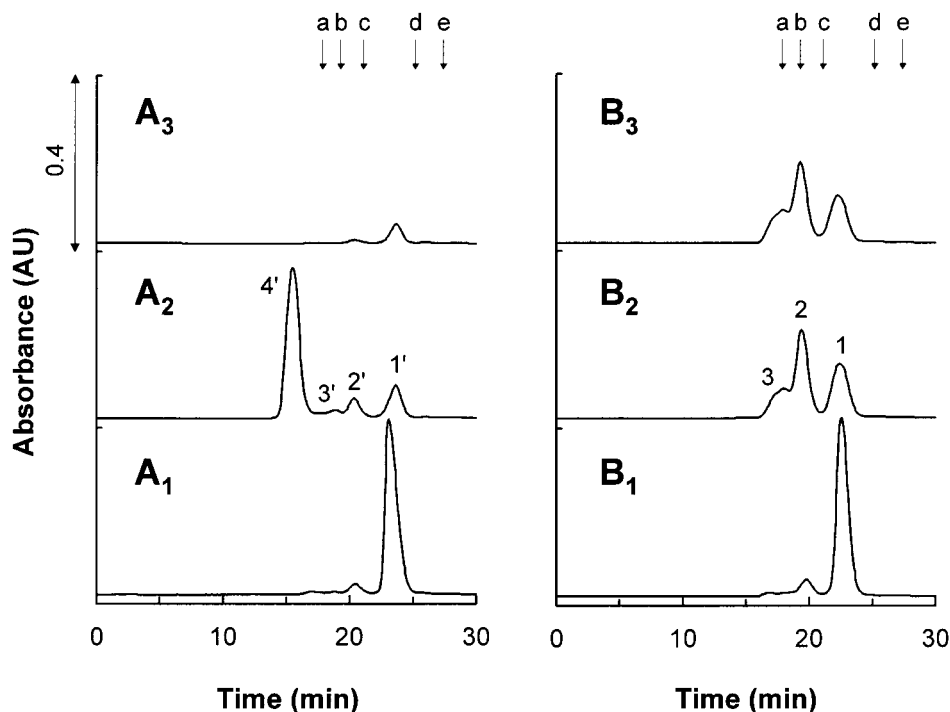
## RESULTS

### Effect of Heating Temperature on Solubility.

The effect of temperature was studied on conjugates with nine lactose residues per monomer of  $\beta$ -LG in comparison with unglycated protein (control). Figure 1 shows the solubility, at pH 4.6, of samples heated for 10 min at various temperatures. As expected, the precipitation of control  $\beta$ -LG increased with increasing temperature from 65 to 90 °C. The proportion of soluble  $\beta$ -LG recovered varied from  $100 \pm 3\%$  at 65 °C to  $11.5 \pm 3\%$  at 90 °C. When lactose- $\beta$ -LG conjugates were heated, cooled, and adjusted to pH 4.6, no significant precipitation occurred whatever the heating temperature (Figure 1). The proportion of recovered soluble material was  $98.5 \pm 7\%$ . The SEC profiles of soluble species in various treated samples are shown in Figure 2. For the control, high temperatures induced a decrease of soluble  $\beta$ -LG without qualitative change of the chromatographic profiles (Figure 2A). In contrast, heating temperature affected the SEC elution profile of glycated samples. Figure 2B shows that the soluble forms were eluted mainly as dimer until 65 °C (peak 1). Above 70 °C, the solubility of the conjugates was associated with the disappearance of the dimeric form and the appearance of a soluble material, called the new species, with an apparent molecular mass larger than that of the dimer (peaks 2 and 3). The area of these two new peaks increased as temperature increased. Figure 3 reports the evolution of SEC profiles of samples before and after heating at 90 °C and after acid precipitation. For glycated sample, the same chromatographic profile was obtained before and after acid precipitation, indicating that the new species were generated during heating (Figure 3B<sub>2</sub>, B<sub>3</sub>). Heat treatment of control protein induced the formation of insoluble species, the



**Figure 2.** SEC profiles of soluble protein after heating for 10 min at various temperatures: (A) control  $\beta$ -LG; (B) lactose- $\beta$ -LG conjugates. Peaks: 1, dimer; 2 and 3, newly formed species. Arrows indicate molecular mass markers: (a) aldolase; (b) human serum albumin; (c) ovalbumin; (d)  $\alpha$ -chymotrypsinogen; (e) ribonuclease A.

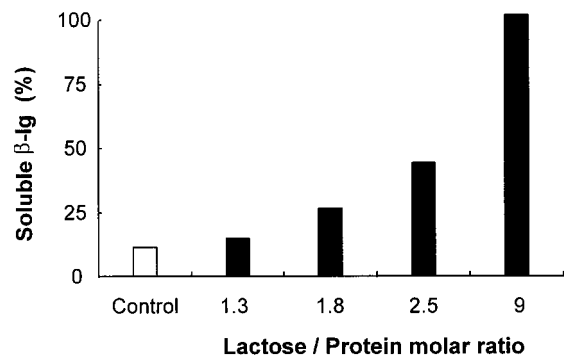


**Figure 3.** Comparison of SEC profiles of control (A) and lactose- $\beta$ -LG conjugates (B) before heat treatment ( $A_1$ ,  $B_1$ ), after heating for 10 min at 90 °C ( $A_2$ ,  $B_2$ ), and after precipitation at pH 4.6 of heated samples ( $A_3$ ,  $B_3$ ). Arrows indicate molecular mass markers (see Figure 2).

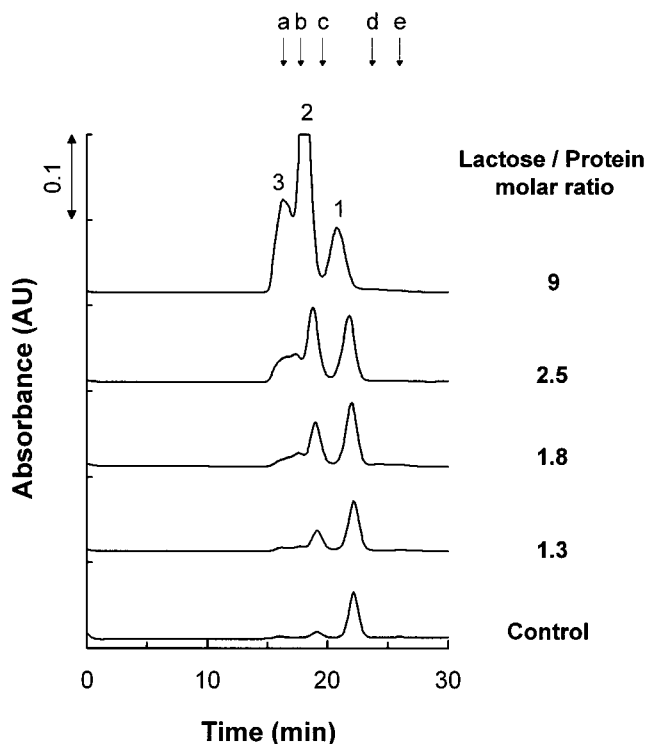
main of which were eluted in the void volume (peak 4', Figure 3A<sub>2</sub>).

**Solubility as a Function of the Lactose/Protein Molar Ratio.** To elucidate whether the number of bound lactose molecules affects the solubility, as well as the relative proportion of peaks 1–3,  $\beta$ -LGs with increasing numbers of bound lactose were used. The results reported in Figure 4 indicate that, for the control

sample, 11.5  $\pm$  3% of the protein was recovered in the soluble form, eluted mainly as a dimer (Figure 5). When the conjugates were used, the proportion of soluble material, after heating at 90 °C, increased with the increase in the lactose/protein molar ratio, to reach  $\sim$ 95  $\pm$  4% for the sample with nine lactose molecules bound per monomer of  $\beta$ -LG. The percentage of the new induced species in the SEC chromatogram (peaks 2 and



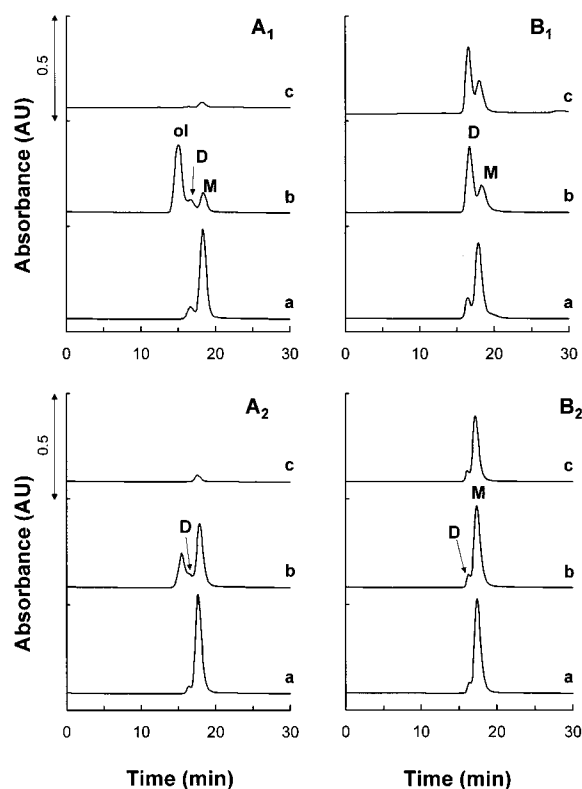
**Figure 4.** Solubility at pH 4.6 of  $\beta$ -LG heated at neutral pH as a function of lactose/protein molar ratio. Control and glycosylated  $\beta$ -LG samples at a concentration of 3 mg/mL were treated for 10 min at 90 °C, pH 7.4, and then precipitated at pH 4.6.



**Figure 5.** SEC profiles of soluble control  $\beta$ -LG and lactose- $\beta$ -LG conjugates. The number of bound lactose per  $\beta$ -LG monomer is indicated. Conditions are as described in Figure 4. Peaks: 1, dimer; 2 and 3, newly formed soluble species. Arrows indicate molecular mass markers (see Figure 2).

3, Figure 5) also increased as a function of the lactose/protein ratio and became dominant for samples with 2.5 and 9 lactose residues attached per molecule of  $\beta$ -LG. Hence, these new species could be responsible for maintaining the high solubility of glycosylated samples.

**Preliminary Characterization of Soluble Material.** *SEC under Dissociating Conditions.* Figure 6 shows the SEC elution profiles of heated control and glycosylated samples in the presence of 6 M urea  $\pm$  DTT, before (samples b) and after precipitation at pH 4.6 (samples c). Under these analytical conditions, unheated glycosylated and control  $\beta$ -LGs were eluted mainly as a monomer (Figure 6, samples a). As expected, the heated control showed the presence of various species, including monomers, dimers, and high molecular mass oligomers (Figure 6A<sub>1</sub>b). The major part of these oligomers was dissociated into monomers after treatment with 10 mM DTT (Figure 6A<sub>2</sub>b). Precipitation of heated control at



**Figure 6.** SEC elution profiles of control (A) and glycosylated (B)  $\beta$ -LG solutions treated in 6 M urea (A<sub>1</sub>, B<sub>1</sub>) or in 6 M urea + 10 mM DTT (A<sub>2</sub>, B<sub>2</sub>): (a) unheated samples; (b) samples heated for 10 min at 90 °C; (c) acid soluble fractions of heated samples. SEC analysis was performed in 0.1 M Tris-HCl buffer/0.15 M NaCl, pH 8, containing 6 M urea. Abbreviations: ol, oligomers; D, dimers; M, monomers.

pH 4.6 led to a drastic decrease of soluble material (Figure 6A<sub>1</sub>c, A<sub>2</sub>c). In contrast, the glycosylated sample showed mainly the dimeric forms, and no species larger than dimers were detected either in whole heated sample (Figure 6B<sub>1</sub>b) or after precipitation at pH 4.6 (Figure 6B<sub>1</sub>c). The observed dimeric forms, resistant to urea, were almost completely dissociated after treatment with DTT in whole as well as in precipitated samples (Figure 6B<sub>2</sub>b, c). The formation of stable linked dimers between glycosylated  $\beta$ -LG molecules was confirmed by SDS-PAGE analyses of heated samples (results not shown).

*Sensitivity to Trypsin Digestion.* The sensitivity of heat-induced species of both control and glycosylated samples to trypsin digestion was monitored by the evolution of the corresponding peaks separated in the SEC (Figure 3, samples A<sub>2</sub> and B<sub>2</sub>). The percents of residual area of each peak in heated control and glycosylated  $\beta$ -LG were compared after various digestion times (Table 1). Under the experimental conditions applied, 2 h was needed to digest 50% of the dimer form (peak 1') and 63% of the aggregates (peak 4') in the control sample. In comparison, the newly formed species in the glycosylated sample (peaks 2 and 3) showed a relatively high sensitivity to enzyme digestion. Digestions of 42 and 70% of peak 2 were obtained, respectively, after 5 and 120 min, whereas 70 and 96% of peak 3 disappeared during these two periods. On the other hand, the dimeric form (peak 1) in the glycosylated  $\beta$ -LG, which represented ~50% of the initial total area, seemed to be resistant to enzyme degradation.



**Table 1. Comparison of Trypsin Digestion of Various Peaks Corresponding to Heat-Induced Species in Control (Figure 3A<sub>2</sub>) and Glycated  $\beta$ -LG (Figure 3B<sub>2</sub>)<sup>a</sup>**

digestion time (min)	residual area (%)						
	control $\beta$ -LG				glycated $\beta$ -LG		
	peak 1' <sup>b</sup>	peak 2'	peak 3'	peak 4'	peak 1	peak 2	peak 3
0	100	100	100	100	100	100	100
5	91	99	62	73	152	58	30
15	93	86	54	64	143	27	12
120	51	49	33	37	98	30	4

<sup>a</sup> Samples were heated for 10 min at 90 °C, and the hydrolysis was performed at 40 °C at an enzyme/substrate molar ratio of 1:140. <sup>b</sup> See Figure 3 for peak identification.

## DISCUSSION

The results of this study clearly indicate that covalent attachment of lactose molecules to  $\beta$ -LG resulted in enhanced solubility of the glycoconjugates at pH 4.6 following heat treatment (65–90 °C for 10 min) at pH 7.4 and 0.116 M NaCl compared with the control heated samples. Although Arakawa and Timasheff (1982) reported that the presence of a high concentration of lactose in the medium increased the heat stability of  $\beta$ -LG, no heat stabilization effect of free lactose at 46 g/L was observed under our conditions (results not shown). The observed enhancement of the solubility of glycated  $\beta$ -LG was associated with the transition from the native dimeric state to new species exhibiting higher apparent molecular masses induced during heating. The proportion of the newly formed soluble species increased as a function of both temperature and number of lactose residues bound per  $\beta$ -LG molecule. These results suggest that bound lactose stabilizes an intermediate state of the denaturation process which is still soluble at pH 4.6.

Previous work has established that thermal denaturation of  $\beta$ -LG proceeds via a number of steps involving the reversible dissociation of the dimer to monomers, unfolding of monomers, and aggregation of unfolded molecules, via hydrophobic interactions and sulfhydryl–disulfide interchange reactions (Sawyer, 1968; Iametti et al., 1996). During this overall denaturation mechanism, Iametti et al. (1996) showed that an activated monomer with an exposed reactive –SH group and adhesive hydrophobic surfaces played a primary role. As shown here, stabilization of the covalent dimer state indicates that bound lactose does not decrease the reactivity of the free sulfhydryl group which became exposed after heating. However, the polymerization steps of  $\beta$ -LG, which involve hydrophobic interactions of unfolded molecules and their subsequent sulfhydryl–disulfide interchange reactions, were limited, probably because of the highly hydrophilic character of the attached lactose residues and steric hindrance. On the other hand, taking into account that protein solubility is related to the hydrophilicity/hydrophobicity balance at the surface of the macromolecule (Damodaran, 1997), the large number of hydrophilic residues linked per formed dimer could explain the observed solubility at pH 4.6.

According to their elution times in SEC, the newly formed soluble species could result from increases in molecular mass (polymerization) and/or molecular volume of the individual protein molecules. SEC analysis, in the presence of urea and DTT, indicated that stabilized dimer was cross-linked via disulfide bonds. The

observed sensitivity to DTT also ruled out the covalent intermolecular cross-linking through Maillard reaction products. This reaction was previously reported to occur during long-term storage of a sugar–protein mixture, for example, lactulose- $\beta$ -LG or lactulose-ovalbumin (Matsuda et al., 1991; Kato et al., 1989). Consequently, the soluble new species eluted before the native dimer (peaks 2 and 3 in the SEC profiles of glycated  $\beta$ -LG) would correspond either to the unfolded dimers or to small oligomers formed by their subsequent noncovalent polymerization. The high susceptibility to trypsin digestion seems to be in favor of unfolded dimers with a less compact and more expanded conformation than the native globular dimer. More extensive structural study is currently being undertaken to complete the biochemical characterization of the newly formed soluble species.

## ACKNOWLEDGMENT

We thank J. Fauquant for the preparation of native  $\beta$ -lactoglobulin.

## LITERATURE CITED

- Arakawa, T.; Timasheff, N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536–6544.
- Burr, R.; Moore, C. H.; Hill, J. P. Evidence of multiple glycosylation of bovine  $\beta$ -Lactoglobulin by electrospray ionisation mass spectrometry. *Milchwissenschaft* **1996**, *51*, 488–492.
- Damodaran, S. Food proteins: an overview. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Dekker: New York, 1997; pp 1–24.
- de Wit, J. N.; van Kessel, Th. Effects of ionic strength on the solubility of whey protein products. A colloid chemical approach. *Food Hydrocolloids* **1996**, *2*, 143–149.
- Hattori, M.; Ogino, A.; Nakai, H.; Takahashi, K. Functional improvement of  $\beta$ -lactoglobulin by conjugating with alginate lyase-lysate. *J. Agric. Food Chem.* **1997**, *45*, 703–708.
- Hoffmann, M. A. M.; Sala, G.; Olieman, C.; de Kruif, K. G. Molecular mass distributions of heat-induced  $\beta$ -lactoglobulin aggregates. *J. Agric. Food Chem.* **1997**, *45*, 2949–2957.
- Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of  $\beta$ -lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106–112.
- Kato, Y.; Matsuda, T.; Kato, N.; Nakamura, R. Maillard reaction of disaccharides with protein: suppressive effect of nonreducing end pyranoside groups on browning and protein polymerization. *J. Agric. Food Chem.* **1989**, *37*, 1077–1081.
- Kitabatake, N.; Cuq, J. L.; Cheftel, J. C. Covalent binding of glycosyl residues to  $\beta$ -lactoglobulin: effects on solubility and heat stability. *J. Agric. Food Chem.* **1985**, *33*, 125–130.
- Léonil, J.; Mollé, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl  $\beta$ -lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **1997**, *80*, 2270–2281.
- Matsuda, T.; Kato, Y.; Nakamura, R. Amino carbonyl reaction of  $\beta$ -lactoglobulin with lactose. *J. Agric. Food Chem.* **1991**, *39*, 1201–1204.
- Morgan, F.; Léonil, J.; Mollé, D.; Bouhallab, S. Nonenzymatic lactosylation of bovine  $\beta$ -lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413–417.
- Morgan, F.; Léonil, J.; Mollé, D.; Bouhallab, S. Modification of bovine  $\beta$ -lactoglobulin by glycation in a powdered state or in an aqueous solution: Effect on association behavior and protein conformation. *J. Agric. Food Chem.* **1999**, *47*, 83–91.

- Nielsen, B. T.; Singh, H.; Latham, J. M. Aggregation of bovine  $\beta$ -lactoglobulin A and B on heating at 75 °C. *Int. Dairy J.* **1996**, *6*, 519–527.
- Persson, K. M.; Gekas, V. Factors influencing aggregation of macromolecules in solution. *Process Biochem.* **1994**, *29*, 89–98.
- Roefs, S. P. F. M.; de Kruif, K. G. A model for the denaturation and aggregation of  $\beta$ -lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883–889.
- Sawyer, W. H. Heat denaturation of bovine  $\beta$ -lactoglobulins and relevance of disulfide aggregation. *J. Dairy Sci.* **1968**, *51*, 323–329.

Waniska, R. D.; Kinsella, J. E. Glycosylation of  $\beta$ -lactoglobulin and surface active properties. In *Food Proteins*; Kinsella, J. E., Soucie, W. G., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; Vol. 99, pp 100–131.

Received for review March 11, 1998. Revised manuscript received January 25, 1999. Accepted January 26, 1999. This work was supported by Diépal-nsa.

JF9802474